PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

B3

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A01H 5/00, C12N 15/00, 1/00

A1

(11) International Publication Number: WO 91/1511

(43) International Publication Date: 17 October 1991 (17.10.9)

(21) International Application Number: PCT/US91/01946

(22) International Filing Date: 22 March 1991 (22.03.91)

(30) Priority data:
502,315 30 March 1990 (30.03.90) US

(71) Applicant: PRESIDENT AND FELLOWS OF HARVARD COLLEGE [US/US]; 17 Quincy Street, Cambridge, MA 01701 (US).

(72) Inventors: LEDER, Philip ; 25 Aston Road, Chestnut Hill, MA 02167 (US). ORNITZ, David, M. ; 20 Harrison Street, #2, Brookline, MA 02146 (US). MOREADITH, Randall, W. ; One Auburn Court, Brookline, MA 02146 (US).

(74) Agent: CLARK, Paul, T.; Fish & Richardson, 225 Franklin Street, Boston, MA 02110-2804 (US).

(81) Designated States: AT (European patent), BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FI (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: A BINARY GENETIC SYSTEM TO CONTROL EXPRESSION OF A TRANSGENE IN A TRANSGENIC ANIMAL

(57) Abstract

A transgenic non-human vertebrate animal having cells containing a transgene either (1) encoding a non-vertebrate transactivator protein, or (2) including a DNA sequence capable of binding the non-vertebrate transactivator protein, and a method of using such animal.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	**ES**	Spain	**MG**	Madagascar
AU	Australia	**FI**	Finland	**ML**	Mali
BB	Barbados	**FR**	France	**MN**	Mongolia
BE	Belgium	**GA**	Gabon	**MR**	Mauritania
BF	Burkina Faso	**GB**	United Kingdom	**MW**	Malawi
BG	Bulgaria	**GN**	Guinea	**NL**	Netherlands
BJ	Benin	**GR**	Greece	**NO**	Norway
BR	Brazil	**HU**	Hungary	**PL**	Poland
CA	Canada	**IT**	Italy	**RO**	Romania
CF	Central African Republic	**JP**	Japan	**SD**	Sudan
CG	Congo	**KP**	Democratic People's Republic of Korea	**SE**	Sweden
CH	Switzerland			**SN**	Senegal
CI	Côte d'Ivoire	**KR**	Republic of Korea	**SU**	Soviet Union
CM	Cameroon	**LI**	Liechtenstein	**TD**	Chad
CS	Czechoslovakia	**LK**	Sri Lanka	**TG**	Togo
DE	Germany	**LU**	Luxembourg	**US**	United States of America
DK	Denmark	**MC**	Monaco		

BNSDOCID: <WO___9115111A1_I_>

A BINARY GENETIC SYSTEM TO CONTROL EXPRESSION OF A TRANSGENE IN A TRANSGENIC ANIMAL

Background of the Invention

The field of the invention is transgenic animals.

Survival and breeding of transgenic animals, particularly those that may be used as disease models, is often precarious due to the deleterious effect of the expressed transgene. For example, in a transgenic strain bearing an oncogene, the animals may develop tumors before they reach reproductive age. Alternatively, an animal having an immune system that is defective because of the presence of a toxic gene may be difficult to maintain because of its susceptibility to infectious disease. Other transgenes may render the transgenic animal sterile.

One method of ensuring maintenance of a strain bearing a particular toxic transgene is to reduce the fixed level of expression of the transgene to a level tolerated by the animal. This has been accomplished in one transgenic system by incorporating one or more copies of a lac operator-like sequence into the promoter region of the transgene, resulting in a permanent, dose-dependent attenuation of expression of that transgene (Tepper et. al., manuscript in preparation). Also, the choice of the promoter region itself offers some degree of control over the particular tissue(s), and, to a very limited extent, the particular developmental stage in which the transgene will be expressed in vivo. For example, expression of a transgene fused to a murine αA-crystallin promoter

region was detected solely in eye lens tissue of an animal bearing the transgene (Overbeek et al., Proc. Natl. Acad. Sci. USA 82:7815-7819, 1985), while an insulin gene promoter was found to direct expression of a transgene specifically to pancreatic ß cells of transgenic mice at embryonic day 10, or to ß cells beginning 10 to 12 weeks after birth (Hanahan, Science 246:1265-1275, 1989). Similarly, a transgene bearing an elastase promoter/enhancer region was expressed specifically in pancreatic aciner cells, beginning several days before birth (Ornitz et al., Nature 313:600-603, 1985). However, the selection of a particular promoter is generally driven by experimental considerations other than optimizing survival of the resulting transgenic line, and may in any case be an inadequate means of attenuating the toxic effects of the gene on the animals.

Summary of the Invention

The binary system disclosed herein allows the indefinite maintenance of a potentially deleterious "target" transgene in a line of transgenic animals by fusing to the target transgene a promoter containing one or more expression control elements [a DNA sequence such as an enhancer or an upstream activating sequence ("UAS")], which promoter is incapable of directing expression of the gene except in the presence of a certain "transactivator protein" which is not endogenous to the animal's species. The term "transactivator protein" herein refers to any polypeptide, naturally occurring or otherwise, which is capable of binding to a specific DNA sequence (such as a UAS) in a gene, and thereby increasing the level of transcription of (or "transactivating") that gene. The combination of a

BNSDOCID: <WO__9115111A1_I_>

given transactivator protein and a DNA sequence to which that protein specifically binds is herein referred to as a "transactivator set". An animal having a transactivated target transgene can then be generated at will, by simply mating an animal bearing the silent target transgene with a second transgenic animal bearing a transgene encoding a transactivator protein capable of activating expression of the target transgene, yielding offspring some proportion of which bear a copy of both of the two transgenes (i.e., they are "bi-transgenic"). If the first mono-transgenic parent is homozygous for the target transgene and the second parent is homozygous for the transactivator transgene, then 100% of their offspring will be bi-transgenic.

The target transgene will be expressed in any tissues of these bi-transgenic offspring in which the appropriate transactivator protein is present at a sufficiently high level: thus, the promoter region associated with the transactivator transgene dictates not only the spactial and temporal pattern of expression of the transactivator transgene, but also, indirectly, the pattern of expression of the target transgene in these bi-transgenic animals. The failure of such bi-transgenic animals to survive and/or reproduce (owing to the toxic effects of the expressed target transgene) is immaterial, as new bi-transgenic animals can be easily generated as needed from the viable stocks of mono-transgenic animal lines.

Even where expression of the target gene is not particularly deleterious to the animal, in some cases it might be desirable to suppress, except on selected occasions, expression of a certain target transgene (for example where such expression would render the animals more aggressive and difficult to handle). In addition,

the binary method disclosed herein provides the advantage of a high degree of flexibility in manipulating the pattern of expression of a given target transgene. For example, one could establish a battery of mono-transgenic transactivator animal lines, each of which lines bears a transgene encoding the same transactivator protein but a different promoter sequence dictating a different pattern of activator transgene expression. The particular pattern of expression desired for a given target transgene can then be achieved by simply selecting the appropriate transactivator transgenic line and mating an animal from that line with an animal bearing the target transgene. Multiple experiments comparing the in vivo effects of various patterns of expression of the target gene can be accomplished very easily and rapidly with such a battery of transactivator transgenic lines, in contrast to the effort expended in generating a series of individual mono-transgenic lines expressing the target transgene in a comparable variety of expression patterns.

Many examples of transactivator proteins (also referred to as "transcriptional factors") have been identified in yeast (such as Saccharomyces cerevisiae) and in a wide variety of other eukaryotic and prokaryotic organisms. One necessary feature of the binary system disclosed herein is that the particular transactivator protein-binding DNA sequence incorporated into the target transgene not be recognized and bound by any transactivator proteins (or other DNA-binding proteins) endogenous to the trangenic animal's species, which proteins might be capable of switching on transcription of the target transgene, or otherwise interfering with the on/off binary system of the invention. To that end, the transactivator set is

ideally derived from an organism that is evolutionarily far removed from the species of the transgenic animal. For example, where the transgenic animal is a vertebrate, the organism from which the transactivator set is derived could be an invertebrate animal (such as an insect), a plant, a single-celled eukaryote, or a prokaryote. The transactivator-binding DNA sequence utilized can be a naturally-occurring sequence (such as any one of the four illustrated in Fig. 2A), or can be a variation on a naturally-occurring sequence (such as in Fig. 2B) which in practice functions to bind the specific transactivator protein in a manner equivalent to the naturally-occurring sequence. The transactivator protein can be a naturally-occurring protein; a mutational variant of such a naturally-occurring transactivator protein, reflecting a mutation (such as a deletion) in the gene encoding the protein, but which retains at least some of the transactivation activity of the naturally-occurring protein; a chimeric protein combining, for example, the DNA-binding domain of one protein (which can be any type of transcription-control protein which binds DNA: e.g., a transactivator protein or a repressor protein such as the E. coli lac repressor) with the transcription-activating region or regions of one or more other proteins; or a synthetic protein in which a DNA-binding peptide is joined to a relatively acidic peptide. GAL4 is one of several known yeast transactivator proteins. Examples of mutational variants of the GAL4 transactivator protein are shown in Fig. 1; either the ß-galactosidase method of Ma and Ptashne (Cell 48:847-853, 1987a) or the chloramphenicol acetyl transferase (CAT) method of Kakidani and Ptashne (Cell 52:161-167, 1988) could be used to assay any other protein with a GAL4 DNA-binding domain for its

usefulness in a GAL4 transactivator set. Furthermore, by substituting a different transactivator-protein-binding sequence for the GAL4-specific DNA sequence utilized by Ma and Ptashne (1987a) and by Kakidani and Ptashne (1988), their methods could be used to assay proteins for use in transgenic transactivator sets other than GAL4-related sets. Ma and Ptashne (Cell 51:113-119, 1987b) found that many highly-acidic amino acid sequences, when linked to a peptide that bound a specific DNA sequence, could stimulate expression in cultured cells from a promoter containing that DNA sequence. Their findings provide some guidance for selecting polypeptides that would be likely to function as transactivator proteins; confirmation of the potential usefulness (of such selected polypeptides) would come by testing them in an in vitro assay similar to the ß-galactosidase assay or the CAT assay referred to above. Transactivator proteins from eukaryotes are likely to be better suited than prokaryotic transactivator proteins for use in the binary system of the invention, because the former are likely to include signal sequences which target their delivery to the nucleus of the cell for binding to genomic DNA. Furthermore, if the transactivator protein is from a eukaryotic organism, a promoter region to which the transactivator protein is known to bind can be excised from its gene and transferred in its entirety into the target transgene to serve as the promoter of the transgene, eliminating the necessity to locate and characterize the precise sequence of DNA to which the transactivator protein binds. This strategy would not be expected to work with a prokaryotic promoter, which would lack many essential features of a functional eukaryotic promoter, such as enhancer elements with

BNSDOCID: <WO___9115111A1_I_>

position independence. However, these disadvantages of a prokaryotic transactivator set can be overcome, and a prokaryotic set employed in the binary trangenic animal of the invention, if (1) the prokaryotic transactivator protein is expressed in sufficient amounts, or is engineered to include a nuclear transport signal, such that enough of it enters the nucleus to stimulate transcription of the target gene; and (2) the relevant prokaryotic transactivator-protein-binding sequence is known, and can be inserted into a target gene which has a promoter that can function properly in a transgenic animal.

In general, the invention features a transgenic non-human vertebrate animal having cells containing a transgene (the "transactivator transgene") encoding a non-vertebrate transactivator protein (i.e., the animal is transgenic for that transgene).

A "transgene" is defined as a piece of DNA which is inserted by artifice (i.e., by a means other than sexual propagation) into a cell, and becomes part of the genome of the animal which develops from that cell. Such a transgene may be a gene which is partly or entirely heterologous (i.e., foreign) to the transgenic animal, or alternatively may represent a gene homologous to a natural gene of the transgenic animal, but which is inserted into the animal's genome at a location which differs from that of the natural homolog. A "transgenic animal" is an animal having cells that contain a transgene, which transgene was introduced into the animal, or an ancestor of the animal, at an embryonic stage. By "embryonic stage" is meant any point from the moment of conception (e.g., as where the sperm or egg bears the transgene) throughout all of the stages of embryonic development of the fetus, and preferably refers to a stage within the first eight days following conception.

By "non-vertebrate transactivator protein" is meant a transactivator protein which occurs naturally in a non-vertebrate organism [e.g., a prokaryote, a single-celled eukaryote (such as yeast), a plant of any type, or an animal (such as an insect) other than a vertebrate]; or which is encoded by a virus other than one which infects vertebrate animals; or which is a genetically engineered transactivator protein (including a mutational variant of a naturally-occurring transactivator protein that does not bind detectably to the genomic DNA of a non-transgenic animal of the same species as the transgenic animal of the invention, but which does bind to the DNA sequence to which the naturally-occurring protein binds).

The invention also features a non-human vertebrate animal which is transgenic for a target transgene that includes a DNA sequence capable of binding the non-vertebrate transactivator protein. In preferred embodiments, the DNA sequence is about 9-35 nucleotides in length; both the transactivator transgene and the target transgene occur in the same (bi-transgenic) animal; and binding by the transactivator protein to the target transgene increases the level of expression of the target transgene: preferably at least 10-fold, and more preferably at least 100 fold, compared to the level of target transgene expression in an animal transgenic only for the target transgene. Most preferably, in the absence of the transactivator protein, the target gene is expressed either not at all, or at a level so low that it approaches the lower limit of detection.

Preferably, the transgenic animal is a mammal (e.g., a rodent such as a mouse); and the transactivator protein contains a DNA-binding domain (i.e., the portion

BNSDOCID: <WO___9115111A1_I_>

of the transactivator protein which is involved in recognizing and binding to a specific DNA sequence) of a transactivator protein derived from a single-celled organism (i.e., a prokaryote, or a lower eukaryote such as a yeast of any kind, including but not limited to Saccharomyces). An example of such a DNA-binding domain is the section of GAL4 which constitutes the 147 amino acid residues nearest to the amino terminus of that protein. There are many other examples in the literature of such DNA-binding domains which have been localized on various transcription-control proteins. The term "transcription-control proteins" covers both positive (e.g., transactivator) and negative (e.g., repressor) transcription-regulating proteins which affect transcription of a gene by binding to a specific DNA sequence associated with that gene.

Examples of transactivator proteins useful in the binary transgenic system of the invention include the many known yeast transactivator proteins, such as GCN4 protein, MATα2 protein, HAP1 protein, PPR1 protein, LAC9 protein, ADR1 protein, and GAL4 protein; mutational variants of these proteins which retain the DNA-binding domain of the wild type protein (such as the biologically-active deletion mutational variants of GAL4 illustrated by solid bars in Fig. 1, in particular the GAL4/236 mutational variant) and proteins genetically engineered to include the DNA-binding domain of wild-type transactivator or other transcription-control proteins. A mutational variant of a given transactivator protein is herein defined as a mutationally altered protein having at least 5% (preferably at least 25%) of the transactivating activity of the wild-type version of the protein as determined by an in vitro transfection assay such as that of Ma and Ptashne (1987a) or Kakidani and Ptashne

BNSDOCID: <WO___9115111A1_I_>

(1988). The DNA-binding domain of a given transactivator protein can be determined by deletion analysis (as by the method of Ma and Ptashne, 1987a) or by extrapolation from the information known about GAL4 and other transactivator proteins (e.g., the N-terminal location and overall positive charge of the DNA-binding domain).

Preferably, the transactivator transgene contains a heterologous promoter region (defined as a 5' untranslated region, including enhancer, which is capable of driving transcription of the transactivator transgene in vivo and is derived from a gene or genes other than the natural gene which encodes the transactivator protein). The choice of a particular heterologous promoter region is dictated by the spatial and temporal pattern of expression that is desired for the transgene, and could include a promoter region which predominantly directs expression in, for example, the reproductive system (e.g., breast, ovary, or testes); the musculoskeletal system (e.g., muscle or joint tissue); the cardiovascular system (e.g., capillaries or heart); the respiratory system (e.g., lung or nasal passages); the urological system (e.g., kidney or bladder); the gastrointestinal system (e.g., pancreas, liver, or intestines); the immune system (e.g., thymus, spleen, or circulating immunological cells); the endocrine system (e.g., pituitary, gonads, and thyroid); the nervous system (e.g., neurons); and the hematopoietic system (e.g., bone marrow and peripheral blood). Specific examples out of the hundreds of possibilities include the elastase promoter region (including its associated enhancer or another functional enhancer), the αA-crystallin promoter region (including enhancer), the insulin promoter region

BNSDOCID: <WO__9115111A1_I_>

(including enhancer), and the albumin promoter region (including enhancer). Viral promoters (such as MMTV LTR and the CMV LTR) which direct expression of viral (or host) genes in specific tissues are other possible choices.

Preferably, the DNA sequence to which the transactivator protein binds is a transactivator protein-binding sequence which is not endogenous to the species of the transgenic animal, in order to reduce or eliminate the ability of endogenous proteins to bind to the DNA sequence. Where the transactivator protein is GAL4 protein or another protein which binds specifically to a GAL4 protein-binding DNA sequence (such as a mutational variant of GAL4 protein), the transactivator-binding DNA sequence in the target transgene is preferably one of the sequences illustrated in Fig. 2.

The invention also includes a vertebrate cell (preferably a mammalian cell that is not a human cell) containing a stably-integrated foreign gene which includes a DNA sequence capable of binding a non-vertebrate transactivator protein, and a method of increasing the level of expression of that foreign gene in the cell. By "stably-integrated foreign gene" is meant a gene which is not endogenous (or a portion of which is not endogenous) to the animal species from which the cell is derived, or which is inserted into the cell's genome at a site that differs from its naturally-occurring site in the genome, and which was originally incorporated into the genome of the cell, or of a predecessor of the cell, by a means other than by sexual crossing (e.g., by transfection into the cell or a predecessor of the cell, by injection into the cell or a predecessor of the cell, by fusion of the cell with a second cell, by generation of a transgenic animal, or by any other asexual means). In preferred embodiments, the

non-vertebrate transactivator protein is directly incorporated into the cell (as by fusion with liposomes carrying the transactivator protein), or is encoded by a second foreign gene which has been introduced into the cell; it is a protein which is naturally produced by a single-celled organism such as a prokaryote or, more preferably, a yeast (or a mutational variant of such a protein); and when bound to the transactivator protein-binding DNA sequence of the foreign gene, it increases the level of expression of the foreign gene in the cell. Examples of such a transactivator protein include all of the examples referred to above.

The invention provides a method of increasing the level of expression of a target transgene in a line of transgenic animals (e.g., vertebrate animals such as mammals, and in particular, rodents, including mice), which method involves mating a first animal from that line of transgenic animals with a second animal which bears (i.e., is transgenic for) a second transgene encoding a non-vertebrate transactivator protein that is capable of transactivating expression of the target transgene. Alternatively, the level of target transgene expression can be increased in an animal transgenic for the target transgene by infecting the animal with a virus, the genome of which has been altered to encode the non-vertebrate transactivator protein. This method would permit targeting of a specific tissue for infection by the virus, and would be unlikely to affect the germline of the animal. An example of a potentially useful virus in a transgenic mouse is the Maloney Murine Leukemia Virus (MoMuLV). The target transgene preferably includes a transactivator protein-binding DNA sequence (such as one of those described above) and the transactivator protein is preferably one of those described above.

BNSDOCID: <WO___9115111A1_I_>

These methods would be particularly useful where it is desireable (for whatever reason) to have both (a) a transgenic animal which expresses the target transgene to a significant degree, and (b) a transgenic animal which is transgenic for but does not express the same target transgene to a significant degree. Such a case might occur where the higher level of expression of the transgene (i.e., the level in a bi-transgenic offspring of the mating) is deleterious to the health of the offspring animal, or otherwise reduces the abilty of the offspring animal to reproduce.

Other features and advantages of the invention will be apparent from the following description of the preferred embodiments, and from the claims.

Description of the Preferred Embodiments

The drawings are first described.

Drawings

Fig. 1 is a representation of a series of deletion mutational variants of the yeast GAL4 protein which were tested for their ability to activate expression from a promoter containing a GAL4 transactivator protein-binding sequence. (A), (B), and (C): relative efficiency of transactivation is quantitated in the final column of each illustration, labelled "ß-gal ACTIVITY". Solid bars indicate what the authors (Ma and Ptashne) termed "those mutants that activate transcription efficiently", while dashed bars indicate poor activation, and open bars indicate no detectable activation (adapted from Tables 1, 2, and 3 of Ma and Ptashne, 1987a). (D): relative efficiency of transactivation is indicated by the intensity of the spot near the top of each lane. pAG4 encodes the full 881-residue GAL4 amino acid sequence, while pAG147

BNSDOCID: <WO___9115111A1_I_>

encodes only residues 1-147, pAG236 encodes GAL4 residues 1-147 and 768-881 (i.e., GAL4/236), and pAG242 encodes GAL4 residues 1-238 and 768-881 (adapted from Figure 2 of Kakidani and Ptashne, 1988).

Fig. 2 is an illustration of (A) four naturally-occurring S.cerevisiae GAL4 transactivator-protein-binding DNA sequences, (B) a synthetic 17 bp oligodeoxynucleotide capable of binding the yeast GAL4 transactivator sequence , and (C) a second synthetic 17 bp oligodeoxynucleotide (termed a "Sca I GAL4 binding site" because it contains a Sca I restriction site; Webster et. al., Cell 52:169-178, 1988). (A) and (B) are adapted from Figure 7 of Giniger et al., Cell 40:767-774, 1985.

Fig. 3 is a representation of (A) the MMTV LTR/GAL4 transactivator gene described in Example 2; (B) the int-2 target gene described in Example 1; and (C) an expanded view of the UAS/elastase promoter of the target gene.

Fig. 4 is an illustration of the structure of

(A) pUAS/elastase/hGH,

(B) pG5BCAT,

(C) pEuPu GAL4,

(D) pGEM GAL4, and

(E) pCMV GAL4.

Fig. 5 is an autoradiogram of a thin layer chromatograph separating the species of chloramphenicol which result from the CAT assay; unacetylated chloramphenicol remains near the left of each lane, while acetylated and bi-acetylated chloramphicol migrate toward the right. A, B, and C represent three independent pools of J558L cells stably transfected with pSV7NEO and pG5BCAT, while the plasmids listed down the right margin of the figure indicate the plasmid that was transiently transfected into each cell sample.

BNSDOCID: <WO___9115111A1_I_>

Fig. 6 is a Northern blot analysis of RNA from various tissues of mono- and bi-transgenic mice, probed with (A) a GAL4 cDNA probe, and (B) an int-2 DNA probe.

Fig. 7 is (A) an illustration of the portion of the UAS/Int-2 transgene spanned by the 330 nt DNA probe used for ribonuclease protection analysis, and the portion of the int-2 mRNA which is protected from RNase digestion by that 330 nt probe; and (B) ribonuclease protection analysis of RNA derived from various tissues of mono- and bi-transgenic mice.

Construction of the binary system

The functionality of the binary transgenic system disclosed herein was tested by constructing a binary transgenic system based upon the S. cerevisea GAL4 transactivator set. A target gene was engineered to include, from the 5' end, a rat elastase promoter (but not its enhancer), into which were inserted four copies of a GAL4-binding oligonucleotide (UAS_G); mouse int-2 cDNA; and a human growth hormone (hGH) gene. Construction of a plasmid bearing this target gene is described in Example 1. Example 2 sets forth the construction of the transactivator gene, in which a mouse mammary tumor virus (MMTV) long terminal repeat (LTR) and 600 bp of 5' untranslated c-HA-ras DNA were fused to a sequence encoding a biologically-active deletion mutational variant of GAL4 (GAL4/236), and finally to an SV40 splice/polyadenylation signal sequence. The ability of the GAL4/236 protein to transactivate a stably-integrated target gene bearing a UAS_G was tested by transfection in an in vitro cell line (Example 3). Following the in vitro test, lines of mice mono-transgenic for the transactivator gene (Example 4) or the target gene (Example 5) were

BNSDOCID: <WO___9115111A1_I_>

generated. Bi-transgenic animals resulting from a cross between a mouse bearing the transactivator transgene and a mouse bearing the target transgene are described in Example 6.

Example 1: Construction of the target gene

A plasmid (the "target plasmid") containing the target gene was constructed as follows:

Four synthetic GAL4 UAS oligonucleotides (Fig. 2C; obtained from M. Hollis; Webster et. al., Cell 52:169-178, 1988), were fused at position -72 of the rat elastase promoter (Swift et al., J. Biol. Chem. 250: 14271-14280, 1984; Figure 3C). The rat elastase promoter was selected because, in the absence of a functional enhancer element, a transgene bearing this promoter is not expressed in transgenic mice, but can be activated by any of a number of heterologous enhancers (Ornitz et al., Molec. Cell. Biol. 7:3466-3472, 1987). This UAS/elastase promoter was then fused upstream of the 2.1 kb human growth hormone (hGH) gene (Seeburg, DNA 1:239-249, 1982). This plasmid (pUAS/elastase/hGH; Fig. 4A) was used as the target plasmid for one of the in vitro experiments described in Example 3. For use in generating the transgenic mouse of Example 5, it was further modified, as follows: a 2 kb Hind III fragment containing the int-2 gene (Dixon et al., Molec. Cell. Biol. 9:4896-4902, 1984; Muller et al., EMBO J. in press, 1990) was blunt ended, ligated to Bql II linkers, cut with Bql II, and cloned into the Bamh I site of UAS/elastase/hGH to yield the target plasmid shown in Fig. 3B. Int-2 is a murine gene encoding a protein, related to fibroblast growth factors, that is implicated in both murine and human breast neoplasia (Dickson et al., Cell 37:529-536, 1984). Normally expressed in specific patterns during early embryonic development,

BNSDOCID: <WO__9115111A1_I_>

int-1 has a known phenotype when overexpressed in breast and prostate tissues of transgenic mice (Muller et al., 1990). The hGH portion of this construct provides introns and polyadenylation sequences, and may increase message stability (Palmiter et al., Cell 50:435-443, 1987); however, being downstream of the int-2 termination codon, it is not translated.

Example 2: Construction of the transactivator genes

Three different transactivator plasmids (plasmids containing a transactivator gene) were engineered, each of which contained GAL4/236 cDNA linked to a different promoter region. pMMTV-GAL4/236-SV40 (MMTV GAL4, Figure 3A) consists of 2.3 kb of the mouse MMTV LTR and 600 bp of 5′ untranslated c-HA-ras (Huang et al., Cell 27:245-255, 1981; Muller et al., Cell 54: 105-115, 1988) fused to a 1 kb Hind III fragment of pAG236 (obtained from Ivan Sodowsky and Mark Ptashne) containing the GAL4/236 gene (Kakidani and Ptashne, Cell 52:161-167, 1988). The 3′ end of the construct contains the 800 bp SV40 splice/polyadenylation signal (Seed, Nature 329:840-842, 1987). Also prepared was a control plasmid, pGEM GAL4, in which the GAL4/236 gene has no promoter and thus would not be expressed (Fig. 4D). Other constructs replace the MMTV LTR and ras sequences of pMMTV-GAL/236-SV40 with the cytomegalovirus (CMV) LTR (pCMV GAL4, Fig. 4E; Boshart et al., Cell 41:521-530, 1985), or an immunoglobulin heavy chain enhancer and promoter (pEuPu GAL4, Fig. 4C; Tepper et al., manuscript in preparation; provided by E. Schmidt).

Example 3: In vitro test of the binary system

An in vitro model for the binary transgenic system was utilized to test whether the GAL4 expression product could transactivate expression of a target gene which had been incorporated into the genome of a cell.

BNSDOCID: <WO___9115111A1_I_>

Rather than the int-2 target gene described in Example 1, the target gene utilized for these in vitro experiments was pG5BCAT (Fig. 4B; provided by Ivan Sodowsky and Mark Ptashne), which carries 5 ScaI Gal4 binding sites (Fig. 2C) linked to a CAT gene. In order to facilitate selection of stably-transfected cells, pSV7NEO (which confers neomycin resistance) was also used (Murphy et al., Proc. Natl. Acad. Sci. USA 83:2939-2943, 1986). The plasmids were purified once on CsCL gradients, and then simultaneously electroporated into approximately 10^6 cells of a plasmacytoma cell line, J558L, at a ratio of 18 µg pG5BCAT to 2 µg pSV7NEO, according to the method of Tepper et al. (Cell 57:503-512, 1989). Three independent stable pools of cells were selected by incubation in 0.8 mg/ml G418 (GIBCO), a neomycin analog, for 10 to 14 days. Cells from each of the pools were assayed for chloramphenicol acetyl transferase (CAT) activity by the method of Gorman et al. (Mol. Cell. Biol. 2:1044-1051, 1982). None of the three produced a detectable level of CAT activity. 2×10^7 cells from each pool were then transiently transfected by electroporation with 20µg of one of the following transactivator plasmids: pGEM GAL4, which contains a GAL4/236-encoding sequence with no promoter or transactivator-binding sites (Fig. 4D); pCMV GAL4 (Fig. 4E); or pEuPu GAL4 (Fig. 4C), using the method of Tepper et al., 1989. Following incubation for 36 to 48 hr, protein extracts of the transiently-transfected cell samples were prepared and assayed for CAT activity. As shown in Fig. 5, there is no detectable CAT activity in the control (pGEM GAL4) transfections from any of the three pools of cells: the promoterless GAL4/236 gene in pGEM GAL4 is presumably not expressed, resulting in no GAL4/236 protein to

BNSDOCID: <WO___9115111A1_I_>

transactivate expression of the CAT gene. Transfection with either pCMV GAL4 or pEuPu GAL4, each of which expresses the GAL4/236 protein from a promoter that would be expected to be active in these mammalian cells, produced a significant level of CAT activity in each of the three J558L pools so transfected (Fig. 5), demonstrating that the GAL4/236 transactivating protein was capable of inducing expression from a UAS_G-containing promoter stably incorporated into the genome of mammalian cells.

In another set of in vitro experiments, the UAS/elastase/hGH plasmid described in Example 1 was substituted for the pG5BCAT target plasmid. Cells which were stably transfected with the UAS/elastase/hGH gene produced detectable hGH only if they had also been transiently transfected with a plasmid encoding and expressing GAL4/236 protein.

Example 4: Generation of mice transgenic for the transactivator gene

A NcoI-SpeI restriction fragment containing the MMTV GAL4/236 gene (Fig. 3A) was produced by digesting pMMTV GAL4 with NcoI and SpeI, followed by agarose gel electrophoresis and electroelution of the excised band. The DNA fragment was microinjected into fertilized ova of inbred FVB mice (Taconic Farms, Germantown, NY) by standard techniques (Brinster et al., Proc. Natl. Acad. Sci. USA 82:4438-4442, 1985; see also U.S. Patent No. 4,736,866, herein incorporated by reference). Three transgenic founder animals were identified by hybridizing tail DNA with a 1 kb DNA probe derived from GAL4 cDNA, or a 0.8 kb probe derived from SV40 splice/polyadenylation signal DNA.

BNSDOCID: <WO__9115111A1_I_>

Of these three lines, one line (RW) failed to express the transgene, one line (RX) expressed the transgene at low levels, and one line (RV) expressed the transgene at relatively high levels in several tissues (Table 1). The RV line was chosen for further analysis and matings. To assess the pattern and level of transgene expression in the RV line, RNA from several tissues were analyzed by Northern blot. Figure 6A demonstrates that the GAL4/236 gene is expressed in certain tissues of mice bearing the MMTV GAL4/236 transgene (mice 1, 2, and 4 of Fig. 6A). The prominent 2.4 kb band is the predicted size for the MMTV GAL4/236 mRNA. The GAL4-specific DNA probe hybridized to RNA samples from breast tissue ("Br"), salivary gland ("Sa") and epididymis ("Ep"), but not to samples from liver ("Li"), kidney ("Ki"), or spleen ("Sp") of these animals. This pattern of expression is consistent with the pattern of MMTV LTR-regulated expression in other lines of transgenic mice (Stewart et al., Cell 38:627-637, 1984).

Example 5: Generation of mice transgenic for the target gene

Digestion of the int-2 target plasmid with Hind III yielded a 4.5 kb UAS/int-2 gene-containing DNA fragment that was isolated by electroelution from a band excised from an agarose electrophoresis gel. This fragment was microinjected into fertized FVB mouse ova as described above; six founder mice were identified by hybridization of tail DNA with a 1 kb hGH gene-specific DNA probe. Transgenic mouse lines derived from these six founder mice (designated DG, DH, DX, DY, DZ, and OA) appear to be fertile and free of pathology by both gross and histological examination. No expression of the

BNSDOCID: <WO___9115111A1_I_>

int-2 target gene could be detected in any of these six monogenic transgenic lines by Northern blot analysis, performed by electrophoresing 10 µg of total RNA from each tissue (Chirgwin et al., Biochemistry 18:5294-5299, 1979) on 1.3% agarose gels containing formaldehyde, blotting to nylon membranes (Genescreen; DuPont), and hybridizing with ^{32}P-labeled int-2 specific DNA probes (prepared by random hexamer labelling). See, for example, Fig. 6B, wherein the mouse designated "3" was a pregnant DZ mono-transgenic female, while mouse 5 was a virgin DZ mono-transgenic female. Likewise, RNase protection analysis [performed as described by Melton et al. (Nucl. Acids Res. 12:7053-7056, 1984) by hybridizing 40µg of total RNA from each specified tissue of mice 3 and 5 (and 10-20µg RNA from each of mice 1, 2, and 4) to a 330 nt antisense riboprobe derived from the 5′ end of the int-2 target gene (shown with the expected 188 nt protected fragment in Fig. 7A), digesting with RNase A and RNase T1 (SIGMA), and electrophoresing on 6% acrylamide sequencing gels] did not detect int-2 target gene expression in any of several tissues tested from these two mono-transgenic mice designated 3 and 5 (see Fig. 7B, wherein "tR" denotes tRNA, "Pa" denotes pancreatic tissue, and the other abbreviations have the same meanings as those in Fig. 6).

Example 6: Generation of bi-transgenic animals

Mice from each of the six target transgenic lines were bred to mice from the "RV" transactivator line. Bi-transgenic offspring were identified by hybridization of tail DNA with two DNA probes: one representing hGH DNA (as in Example 5), and one representing either GAL4/236 cDNA or SV40 splice/polyadenylation signal DNA (as in Example 4).

Those offspring which were shown to be bi-transgenic were then assayed (a) for int-2 and GAL4/236 gene expression by mRNA analysis, and (b) for int-2-induced phenotypes.

mRNA Analysis

Northern blot analysis reveals that the pattern of int-2 gene expression in bi-transgenic mice is identical to that of GAL4/236 gene expression in such mice [Table 1; Fig. 6, in which 10µg RNA from each indicated tissue was probed with (A) a GAL4-specific DNA probe, or (B) an int-2-specific DNA probe. Mouse 1 was a virgin DZxRV bi-transgenic female; mouse 2 was a pregnant DZxRV bi-transgenic female; mouse 4 was a DZxRV bi-transgenic male; and mice 3 and 5 were mono-transgenic DZ females, as described in Example 5]. GAL4/236 was expressed at high levels in breast and salivary glands of both virgin and pregnant female bi-transgenic mice, and in salivary glands and epididymis of male bi-transgenic mice. Similarly, int-2 mRNA is present at high levels in these tissues of bi-transgenic animals. The prominent 3 kb band is the predicted size for an int-2/hGH mRNA.

To determine if the GAL4/236-induced int-2 transcript is initiated properly, an RNase protection probe overlapping the elastase promoter and 5' int-2 sequences (Fig. 7A) was hybridized to RNA from several tissues of these mice 1-5. Fig. 7B shows evidence of 185 nt and 188 nt protected fragments in tissues from breast tissue, salivary gland and epididymis of bi-transgenic mice. The larger fragment, 188 nt, maps precisely to position +1 of the rat elastase promoter. This indicates that the GAL4/236 protein interacts with normal cellular transcription factors responsible for proper mRNA initiation.

BNSDOCID: <WO___9115111A1_I_>

Phenotypic Analysis

Control mono-transgenic mice containing only the int-2 target gene appear, upon gross examination, to be normal. Whole mount preparations from breast tissue of int-2 mice are indistinguishable from those of normal, non-transgenic, age-matched FVB mice. Histologically, tissues from both virgin and pregnant int-2 mono-transgenic mice are identical to those of non-transgenic FVB mice. Similarly, the RV mono-transgenic mice exhibit no evidence of pathology either grossly or histologically.

The mammary tissue of virgin bi-transgenic females appears normal, whereas pregnant bi-transgenic mice develop massively enlarged mammary glands owing to int-2 gene expression and increased levels of certain hormones during pregnancy. Within 2 weeks postpartum, the mammary glands regress substantially; however, histological analysis of the glandular tissue at autopsy shows that it remains abnormally hyperplastic. Whole-mount preparations of mammary fat pads reveal abnormal ductal structures in mammary tissue of virgin bi-transgenic mice. Unlike normal ductal structures in control mice, the ducts in these bi-transgenic mice do not extend into the fat pad; furthermore, the ducts in bi-transgenic mice form multiple budding structures and, occasionally, dilated cystic vesicles.

Histologic sections from breast tissue of virgin bi-transgenic females demonstrate mammary ductal structures that are mildly hyperplastic, with some epithelial cells containing lipid droplets characteristic of lactating cells. In breast tissue of pregnant mice the gland is massively hyperplastic, yet fails to exhibit the secretory lipid droplets characteristic of lactating mammary epithelium. In most

cases the mouse is unable to nurse its litter; however, if the litter is maintained by a foster female mouse for several days, lactation can begin in some of the bi-transgenic mice. The phenotype observed in several of these bi-transgenic lines is similar yet more severe than that observed in other transgenic mice containing the MMTV LTR directly fused to the int-2 gene (Muller et al., 1990). This may correlate with substantially higher levels of int-2 expression in the bi-transgenic mice of the invention.

Of five independent target transgenic lines examined, three (DY, DZ, and OA) have identical severe phenotypes when mated to a MMTV/GAL4/236 transactivator mouse, and the other two lines (DG and DX) appear to have milder phenotypes (Table 1).

Male mice from several of the target lines develop epididymal and prostate hyperplasia (Table 1). Although these mice are able to mate, they fail to sire any offspring even after multiple confirmed vaginal plugs. Autopsy on RV x DY bi-transgenic males revealed an enlarged and dilated epididymal structure; a normal-appearing vas deferens; and a highly cystic, enlarged prostate gland. Histologic examination of the prostate showed a glandular tissue that closely resembles the disease benign prostatic hyperplasia common in humans.

In contrast to mono-transgenic mice containing either a transactivator gene or a target gene, the bi-transgenic animals exhibit parotid and sublingual gland enlargement. Histologic analysis reveals hyperplastic salivary gland tissue in bi-transgenic animals.

BNSDOCID: <WO__9115111A1_I_>

BNSDOCID: <WO__9115111A1_I_>

TABLE 1

Line	Transgene	Transgene Expression						Phenotype					
								Tissue hyperplasia				Ability to	Male
		Breast	Sal. Gland	Epididymis	Testes	Kidney	Liver	Breast	Salivary Gland	Epididymis	Prostate	Nurse Litters	Fertility
RV	MMTV GAL4	+++	+++	+++	-	-	-	no	no	no	no	yes	Fertile
RW	MMTV GAL4	-	-	-	-	-	-	no	no	no	no	yes	Fertile
RX	MMTV GAL4	+						no	no	no	no	yes	Fertile
DG	UAS INT-2	-	-	-	-	-	-	no	no	no	no	yes	Fertile
DH	UAS INT-2	-	-	-	-	-	-	no	no	no	no	yes	Fertile
DX	UAS INT-2	-	-	-	-	-	-	no	no	no	no	yes	Fertile
DY	UAS INT-2	-	-	-	-	-	-	no	no	no	no	yes	Fertile
DZ	UAS INT-2	-	-	-	-	-	-	no	no	no	no	yes	Fertile
OA	UAS INT-2	-	-	-	-	-	-	no	no	no	no	yes	Fertile
DG x RV	INT-2, GAL4				-	-	-	Mild				yes	
DH x RV	INT-2, GAL4				-	-	-						
DX x RV	INT-2, GAL4				-	-	-						Fertile
DY x RV	INT-2, GAL4	+++	+++	+++	-	-	-	Severe	yes	yes	yes	no	Sterile
DZ x RV	INT-2, GAL4	+++	+++	+++	-	-	-	Severe	yes			no	Sterile
OA x RV	INT-2, GAL4	+++	+++	+++	-	-	-	Severe	yes			no	Fertile

Use

The binary transgenic system of the invention provides a means for indefinitely maintaining a silent but potentially deleterious transgene in a viable animal line, and yet producing, at will, an offspring of that line in which the transgene is expressed. This system is useful for supplying animals for use as models of human diseases (e.g., for testing proposed new treatments for such diseases, or for studying causative agents). In addition, the binary system can be applied to the development of animals to serve as a source of, for example, medically-important human proteins or transplantable organs.

Other embodiments are within the following claims.

What is claimed is:

1. A transgenic non-human vertebrate animal having cells containing a transgene encoding a non-vertebrate transactivator protein.

2. The animal of claim 1, wherein said animal has cells containing a second transgene comprising a DNA sequence capable of binding said non-vertebrate transactivator protein.

3. The animal of claim 2, wherein binding of said non-vertebrate transactivator protein to said DNA sequence increases the level of expression of said second transgene in said animal.

4. A transgenic non-human vertebrate animal having cells containing a transgene comprising a DNA sequence capable of binding a non-vertebrate transactivator protein.

5. The animal of claim 4, wherein binding of said non-vertebrate transactivator protein to said DNA sequence increases the level of expression of said transgene in said animal.

6. The animal of claim 5, wherein said cells further contain a second transgene encoding said non-vertebrate transactivator protein.

7. The animal of claim 1, claim 2, claim 3, claim 4, claim 5, or claim 6, wherein said animal is a mammal.

8. The animal of claim 7, wherein said mammal is a rodent.

BNSDOCID: <WO__9115111A1_I_>

9. The animal of claim 8, wherein said rodent is a mouse.

10. The animal of claim 1, claim 2, claim 3, claim 4, claim 5, or claim 6, wherein said non-vertebrate transactivator protein contains a DNA-binding domain of a transcription-control protein from a single-celled organism.

11. The animal of claim 10, wherein said single-celled organism is a prokaryote or a yeast.

12. The animal of claim 11, wherein said single-celled organism is a yeast.

13. The animal of claim 12, wherein said transactivator protein is yeast GAL4 protein or a protein containing the DNA-binding domain of said GAL4 protein.

14. The animal of claim 13, wherein said transactivator protein is a mutational variant of said GAL4 protein.

15. The animal of claim 14, wherein said mutational variant of said GAL4 protein is represented by a solid bar in Fig. 1.

16. The animal of claim 15, wherein said mutational variant of said GAL4 protein is GAL4/236.

17. The animal of claim 1, wherein said transgene comprises a heterologous promoter region.

BNSDOCID: <WO___9115111A1_I_>

18. The animal of claim 6, wherein said second transgene comprises a heterologous promoter region.

19. The animal of claim 1 or claim 6, wherein said heterologous promoter region is selected from the group consisting of the following categories of promoter regions: reproductive system-specific, musculoskeletal system-specific, cardiovascular system-specific, respiratory system-specific, urological system-specific, gastrointestinal system-specific, immune system-specific, endocrine system-specific, nervous system-specific, hematopoietic system-specific, and viral.

20. The animal of claim 19, wherein said heterologous promoter region is selected from the group consisting of the MMTV LTR, the CMV LTR, an immunologlobulin promoter/enhancer, an elastase promoter/enhancer, and an albumin promoter/enhancer.

21. The animal of claim 2, claim 3, claim 4, claim 5, or claim 6, wherein said DNA sequence comprises a transactivator protein-binding sequence which is not endogenous to the species of said animal.

22. The animal of claim 21, wherein said DNA sequence is selected from a sequence illustrated in Fig. 2, and said non-vertebrate transactivator protein is selected from GAL4 protein or a mutational variant of GAL4 protein capable of binding to said DNA sequence.

23. A vertebrate cell containing a stably-integrated foreign gene comprising a DNA sequence capable of binding a non-vertebrate transactivator protein.

24. The cell of claim 23, wherein binding of said non-vertebrate transactivator protein to said DNA sequence increases the level of expression of said foreign gene in said cell.

25. The cell of claim 24, wherein said vertebrate cell is a mammalian cell.

26. The cell of claim 24, wherein said vertebrate cell is not a human cell.

27. The cell of claim 24, wherein said transactivator protein is from a single-celled organism.

28. The cell of claim 27, wherein said single-celled organism is a prokaryote or a yeast.

29. The cell of claim 28, wherein said single-celled organism is a yeast.

30. The cell of claim 29, wherein said transactivator protein is yeast GAL4 protein or a mutational variant of said GAL4 protein.

31. The cell of claim 24, wherein said cell further contains a second foreign gene encoding said transactivator protein.

32. A method of increasing the level of expression of a target transgene in a line of transgenic animals bearing said target transgene, said method comprising mating an animal of said line with a second animal bearing a second transgene encoding a non-vertebrate transactivator protein, said non-vertebrate transactivator protein being capable of transactivating expression of said target transgene.

BNSDOCID: <WO___9115111A1_I_>

33. The method of claim 32, wherein said non-vertebrate transactivator protein is, or is a mutational variant of, a transactivator protein from a single-celled organism.

34. The method of claim 33, wherein said single-celled organism is a yeast.

35. The method of claim 34, wherein said non-vertebrate transactivator protein is selected from said GAL4 protein and a mutational variant of said GAL4 protein.

36. The method of claim 35, wherein said mutational variant of said GAL4 protein is GAL4/236.

37. The method of claim 32, wherein said target transgene comprises a DNA sequence which is capable of binding said transactivator protein, and which is not endogenous to the species of said line of transgenic animals.

38. The method of claim 32, wherein it is desireable to have both (a) a transgenic animal which expresses said target transgene to a significant degree, and (b) a transgenic animal which bears but does not express said target transgene to a significant degree.

39. The method of claim 38, wherein the level of expression of said target transgene in an offspring transgenic animal resulting from said mating reduces the ability of said offspring transgenic animal to reproduce.

40. The method of claim 38, wherein the level of expression of said target transgene in said offspring transgenic animal is deleterious to the health of said offspring transgenic animal.

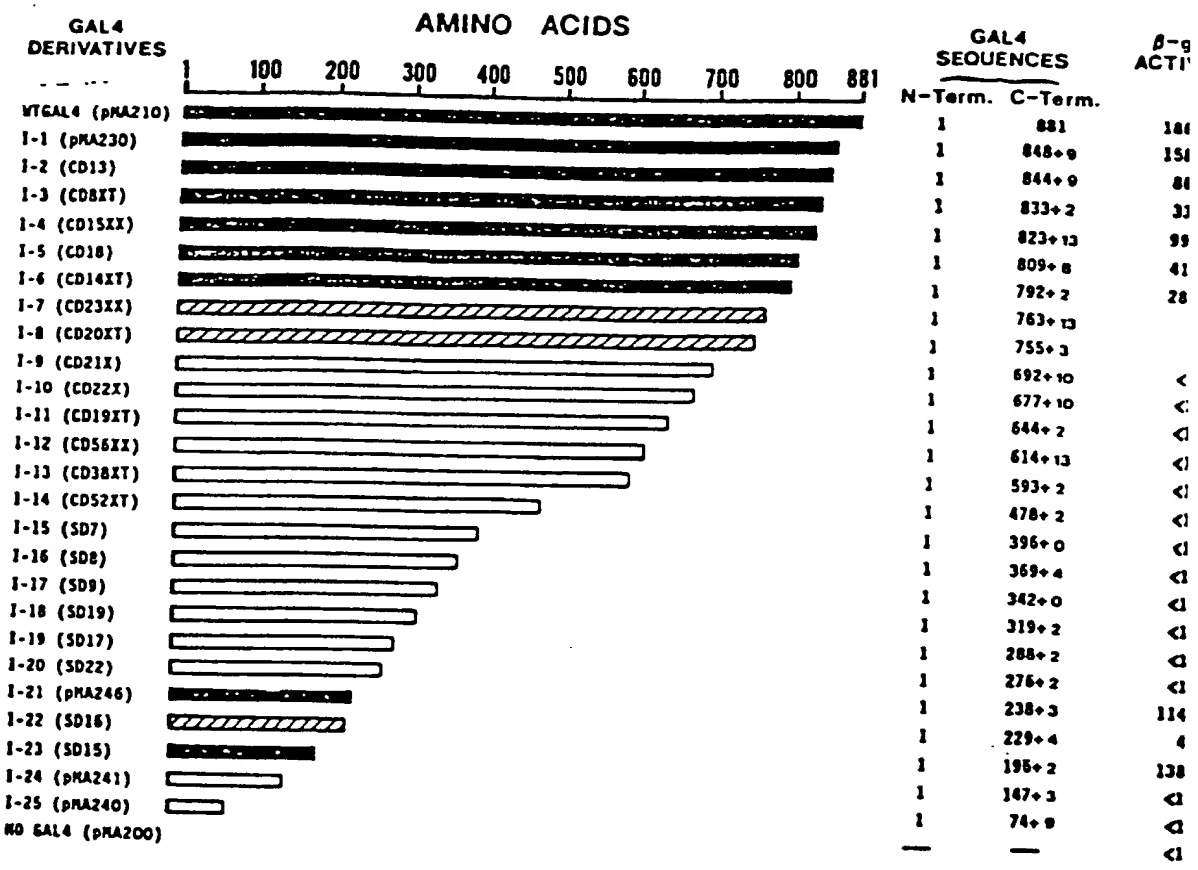
BNSDOCID: <WO___9115111A1_I_>

41. A panel of transgenic non-human vertebrate animals comprising a first animal and a second animal of the same species, said first animal bearing a first transgene comprising a first promoter region fused to a DNA sequence encoding a yeast transactivator protein, and said second transgene comprising a second promoter region fused to a DNA sequence encoding said yeast transactivator protein.

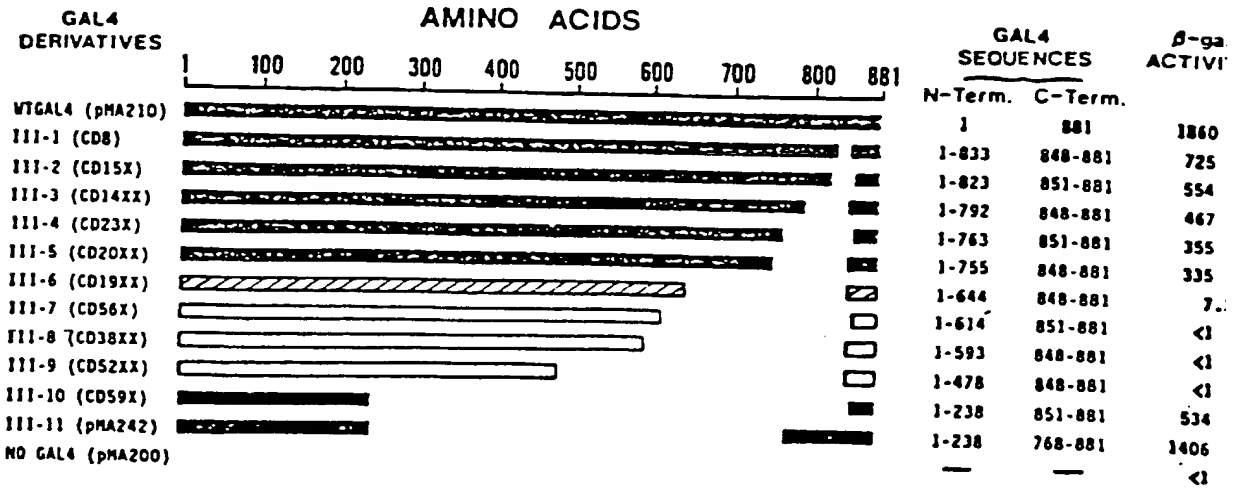
BNSDOCID: <WO__9115111A1_I_>

FIG. 1

(A)



(B)

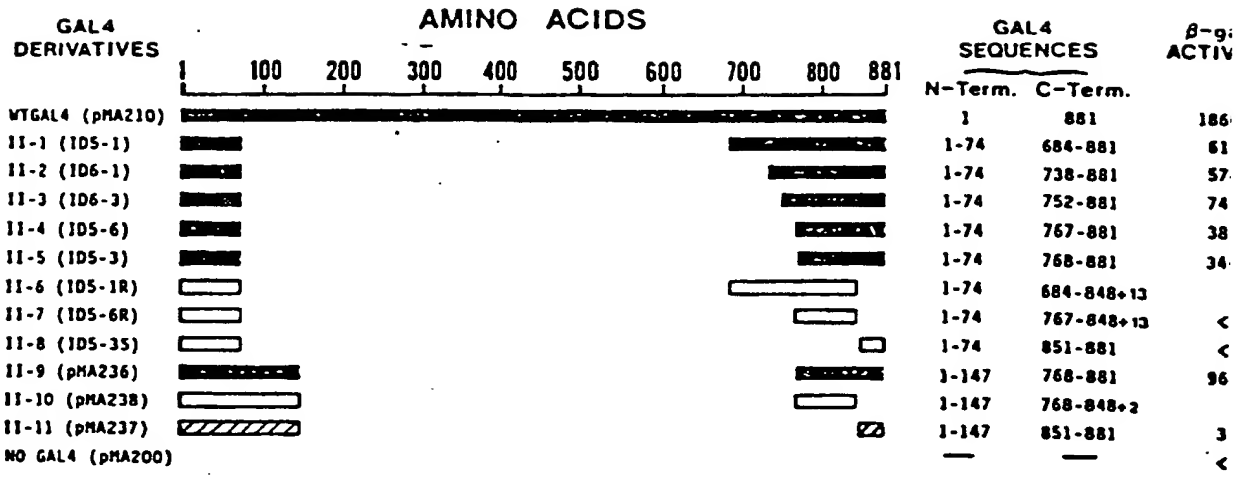


BNSDOCID: <WO___9115111A1_I_>

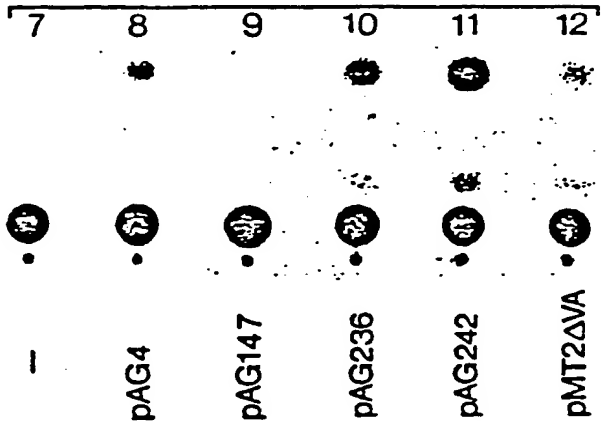
2/11

FIG. 1 (cont.)

(C)



(D)

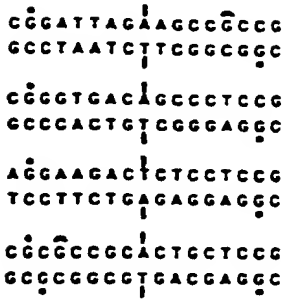


BNSDOCID: <WO__9115111A1_I_>

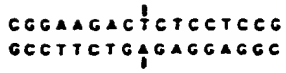
FIG. 2

GAL 4 Binding Sites

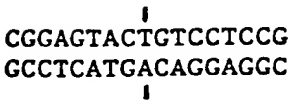
(A)



(B)



(C)



BNSDOCID: <WO___9115111A1_I_>

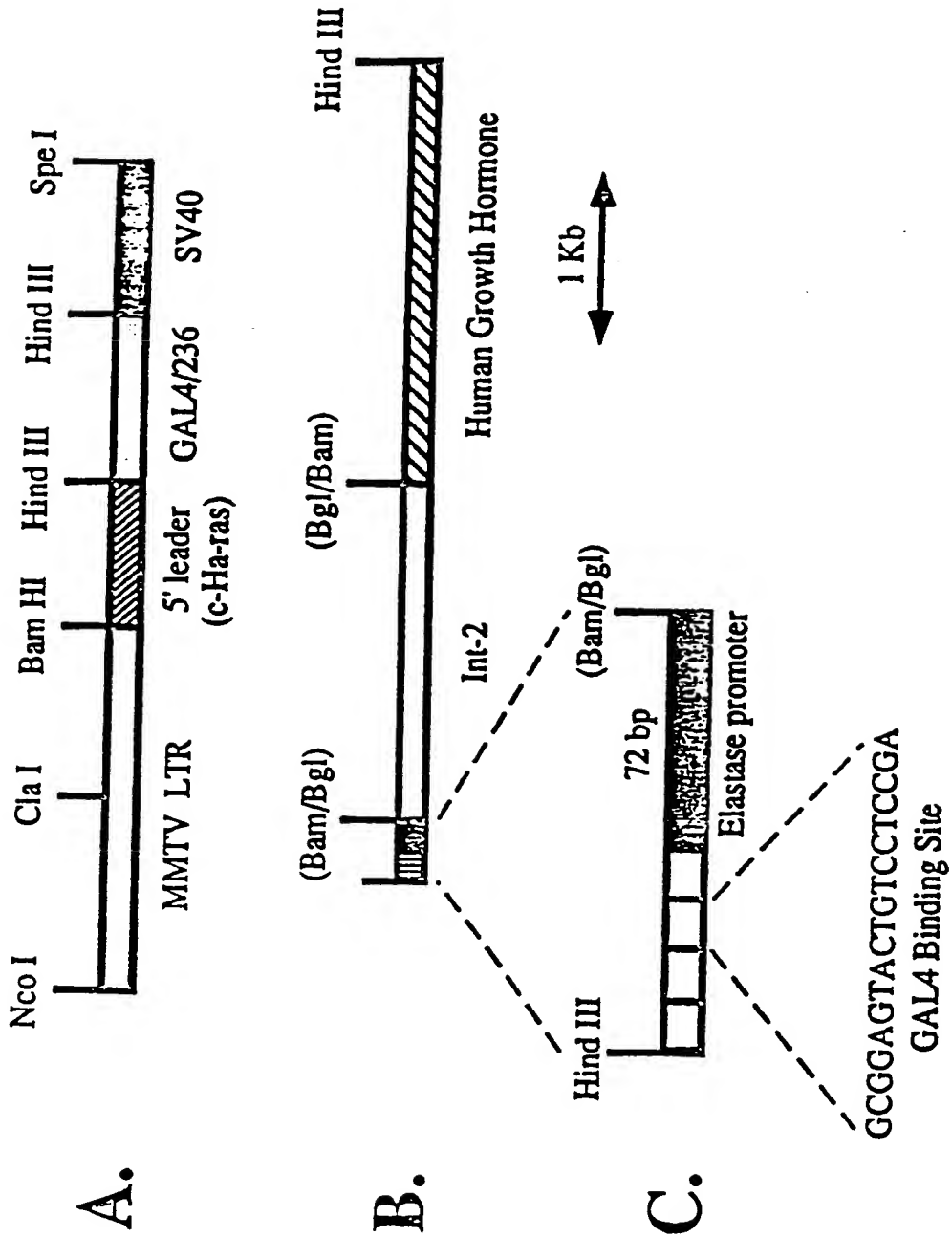
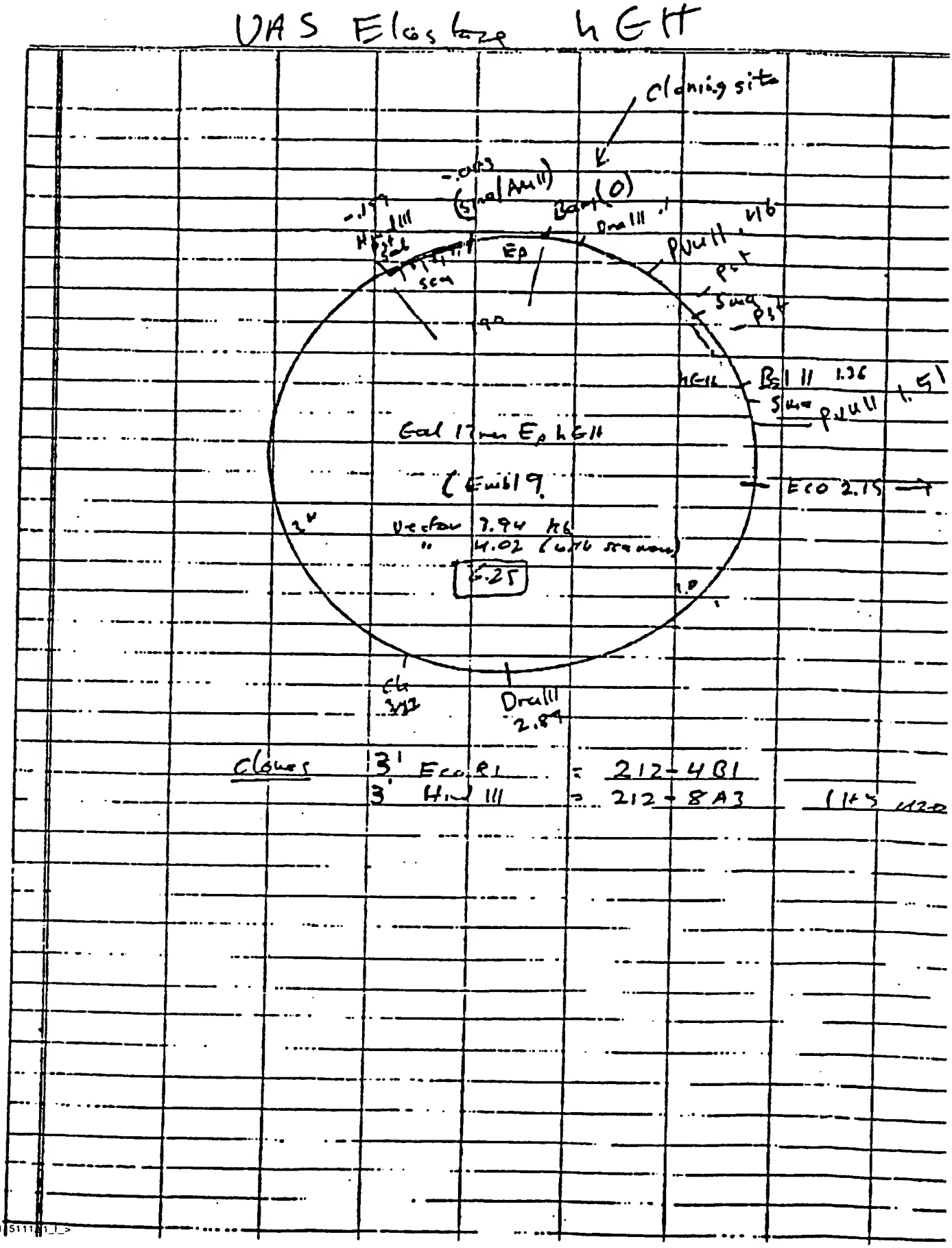


FIG. 3

BNSDOCID: <WO___9115111A1_I_>

5/11

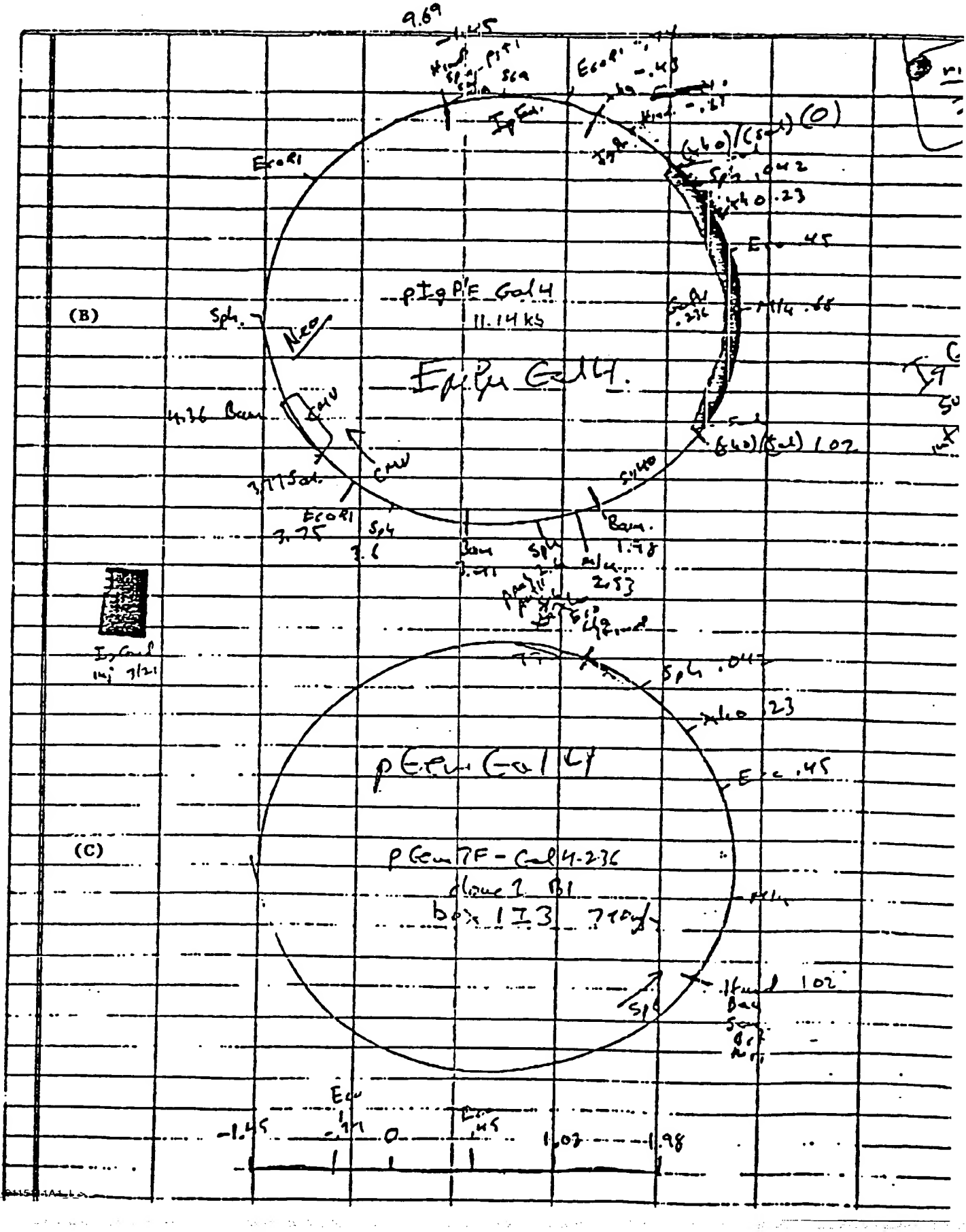
FIG. 4A



BNSDOCID: <WO___9115111A1_I_>

6/11

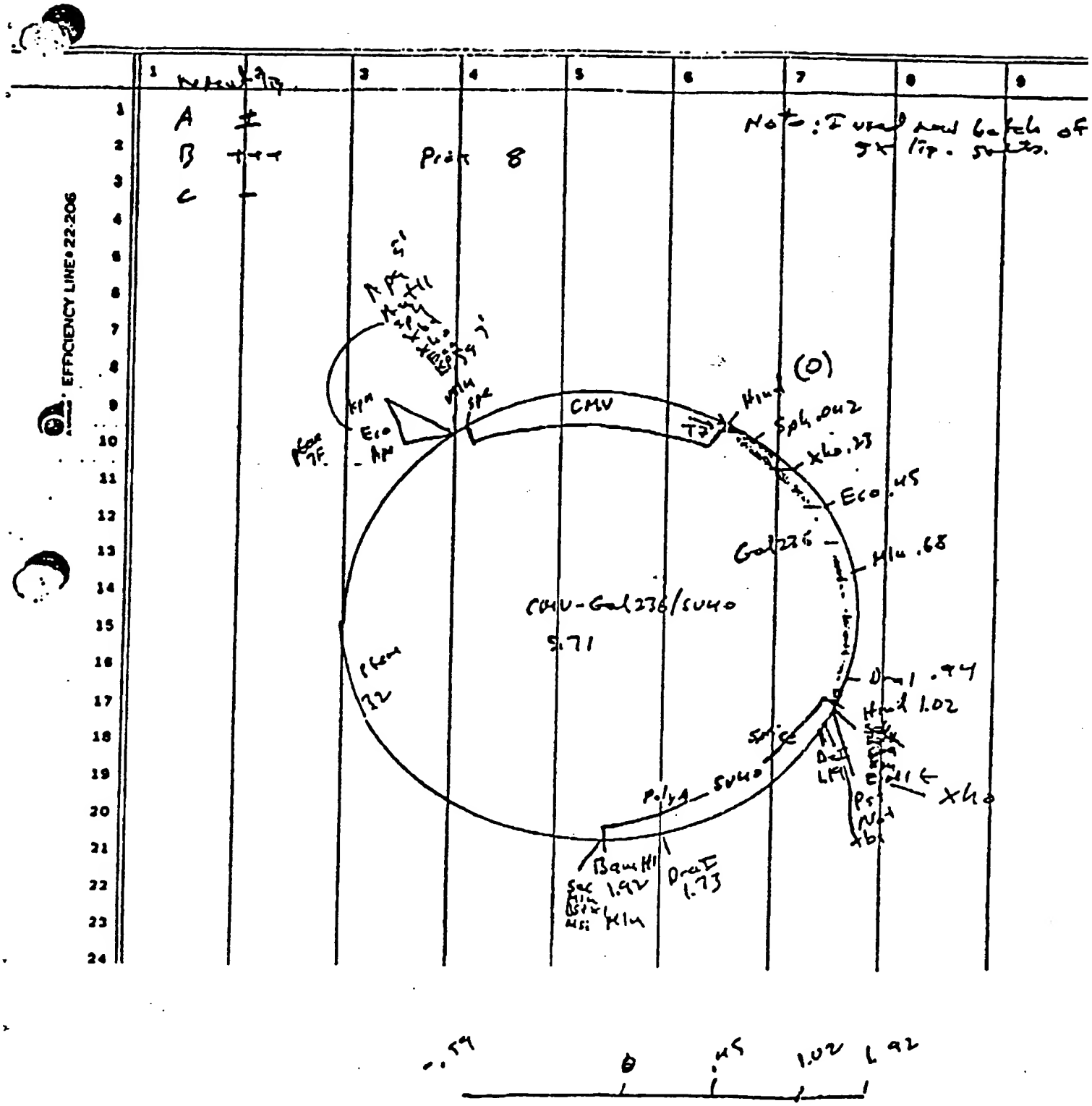
FIG. 4B, 4C



BNSDOCID: <WO_

7/11

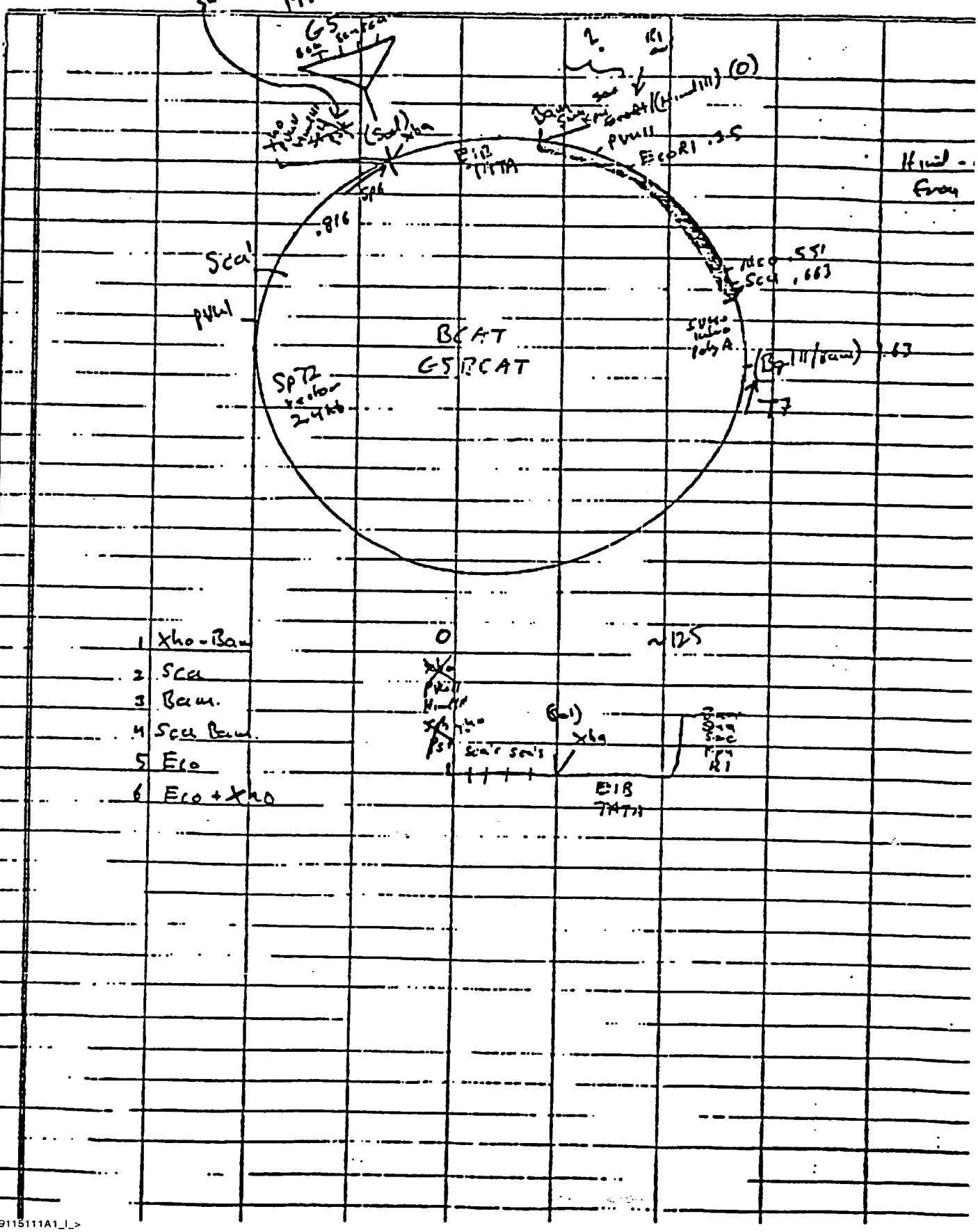
FIG. 4(D)



BNSDOCID: <WO___9115111A1_I_>

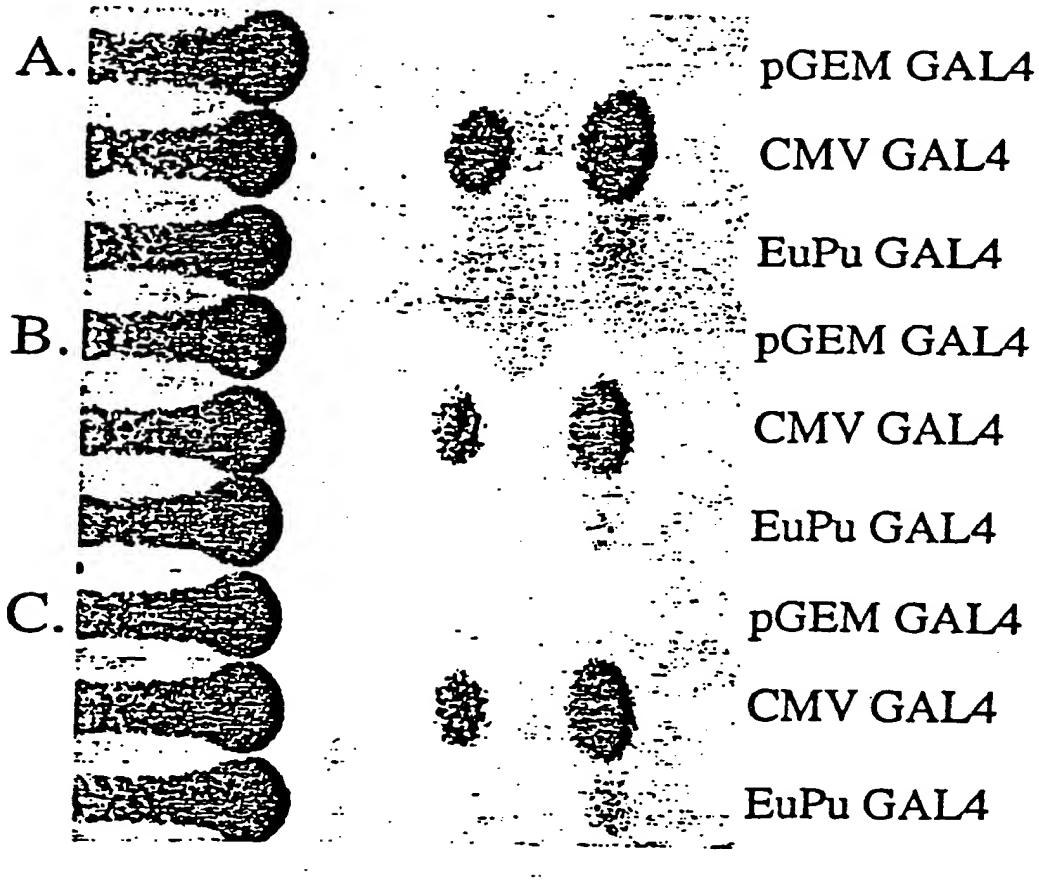
8/11

FIG. 4(E)



BNSDOCID: <WO___9115111A1_I_>

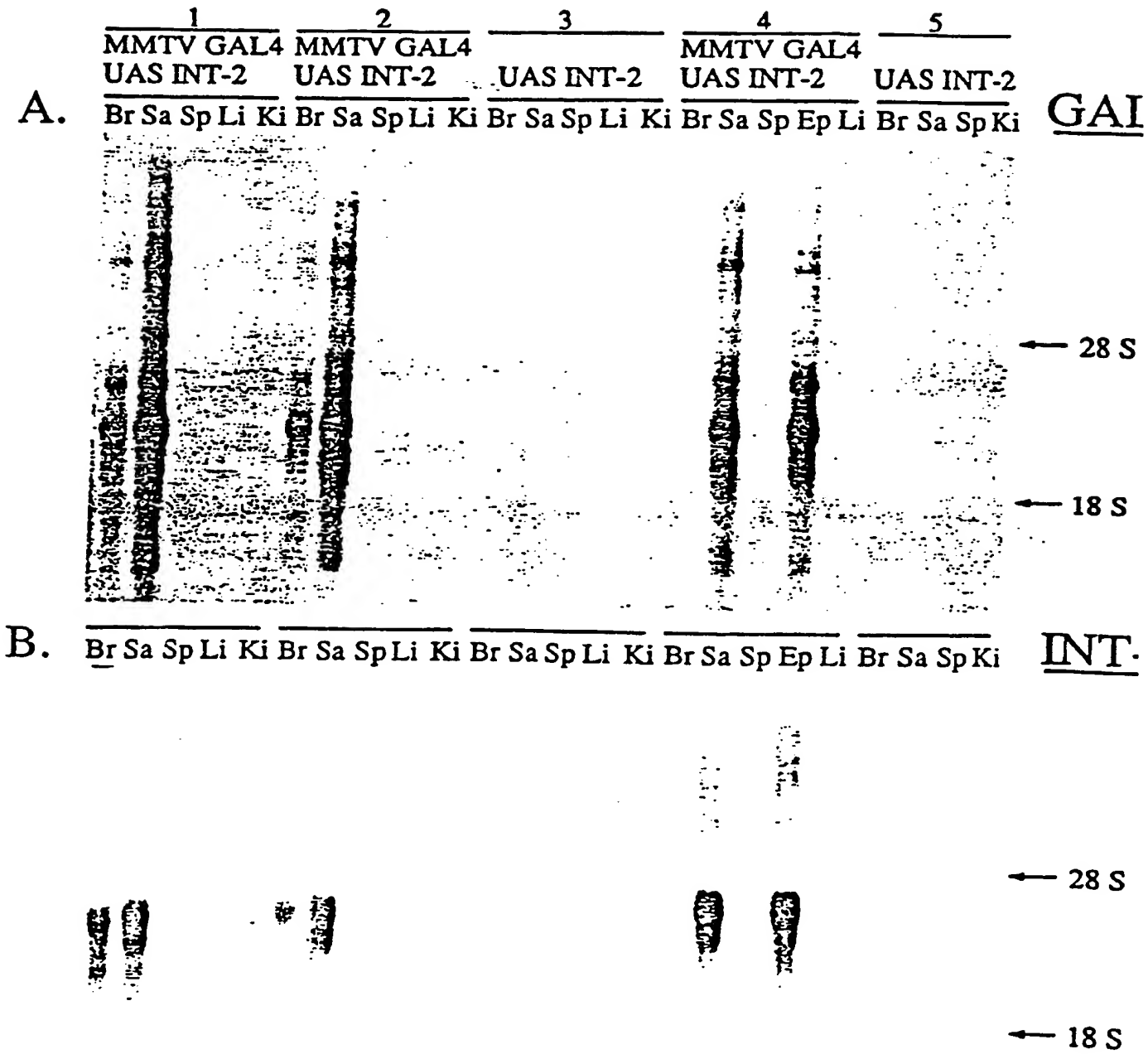
FIG. 5



BNSDOCID: <WO___9115111A1_I_>

10/11

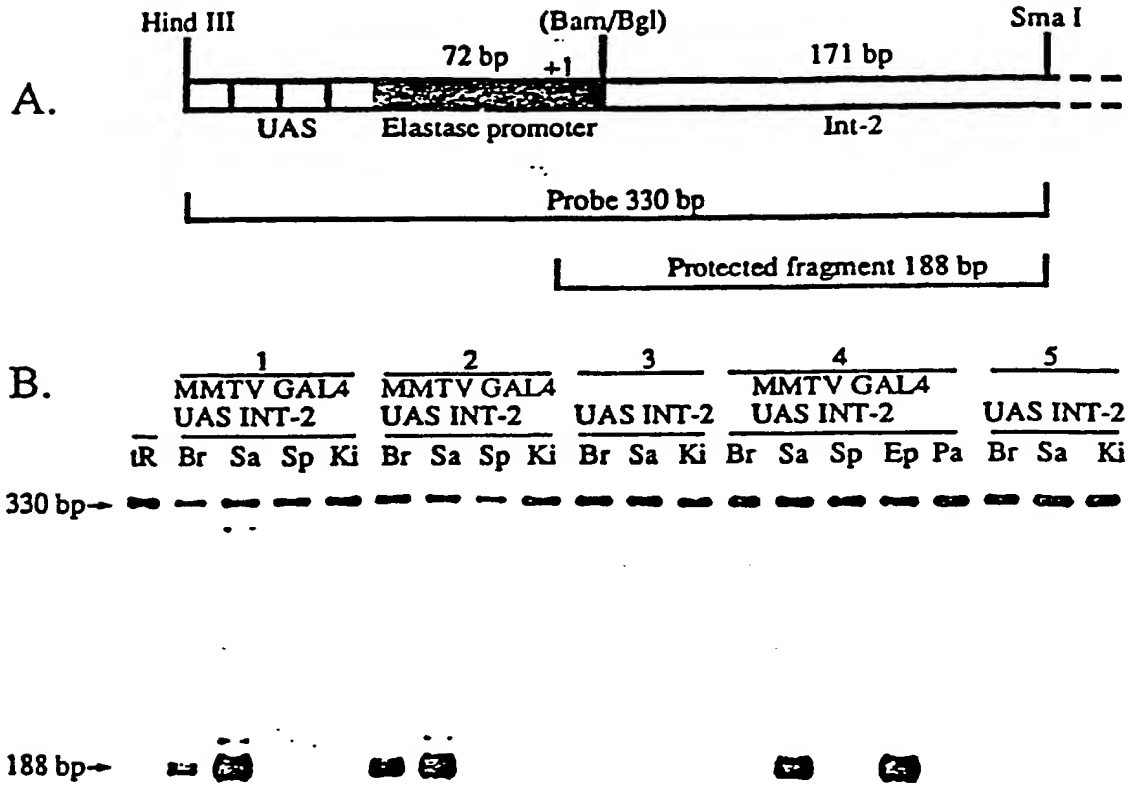
FIG. 6



BNSDOCID: <WO___9115111A1_I_>

11/11

FIG. 7



BNSDOCID: <WO___9115111A1_I_>

INTERNATIONAL SEARCH REPORT

International Application No PCT/US91/01946

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

US CL: 800/2.Dig 1 ; 435/317.1, 172.3; 800/Dig.2,Dig.3,Dig.4,Dig.5

IPC(5): A 01H 5/00; C12N 15/00, 1/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US CL..	800/2, Dig 1-5; 435/317.1, 172.3

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, DIALOG, BIOSIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Proceedings National Academy of Sciences, Volume 86, issued July 1989, G.W. Byrne et al., "Multiplex gene regulation: A two-tiered approach to transgene regulation in transgenic mice", pages 5473-5477, see entire document.	1-9,32,37,38 10-22,33-41
X Y	Nucleic Acids Research, volume 16, number 4, issued 1988, J.S. Khillan et al. "Gene transactivation mediated by the TAT gene of human immunodeficiency virus in transgenic mice", pages 1423-1430, see entire document.	1-9,32,37,38 10-22,33-41

see attached sheet

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 JUNE 1991	21 JUN 1991
International Searching Authority	**Signature of Authorized Officer [11]**
ISA/US	SUZANNE ZISKA

Form PCT/ISA/210 (second sheet) (January 1985)

BNSDOCID: <WO___9115111A1_I_>

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Cell, Volume 52, issued January 1988, N. Webster et al; "The yeast UAS_G is a transcriptional enhancer in human HeLa cells in the presence of the GAL_4 trans-activator," pages 169-178, see entire document.	10-22,33-41
A	Science, Volume 240, issued June 1988, R. Jaenisch, "Transgenic animals", pages 1468-1474, see entire document.	1-22,32-41

BNSDOCID: <WO___9115111A1_I_>

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I, consisting of a first product (a transgenic animal) and a method of using the transgenic aninal, claims 1-22, 32-41
Group II, consisting of a second product (a vertebrate cell containing a stably integrated foreign gene), claims 23-31.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-22,32-41. see telephone memorandum

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BNSDOCID: <WO__9115111A1_I_>